A SURVEY OF

NON-SYMBIOTIC NITROGEN FIXATION IN HAWAIIAN PASTURELANDS

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TABLE OF CONTENTS

Page

LIST OF TABLES

APPENDIX TABLE | Page

LIST OF ILLUSTRATION

INTRODUCTION

Non-symbiotic N_2 fixation has been investigated world-wide and until recently no evaluation of their contribution to Hawaiian soils had been conducted. Although their activity has generally been conceded as low, in pasturelands where no fertilizer is added and where organic matter is allowed to decompose, their contribution to the nitrogen economy of ths soil may be significant. In this study the acetylene-reduction technique was used to determine bi-weekly fixation rates in six different soil types during a period of six months.

Several environmental factors have been shown to govern nitrogenase activity in laboratory studies; however, no assessments have been made of these rate-determining factors as they occur in the field. A stepwise multiple regression analysis of some chemical, microbiological, and climatological factors on nitrogenase activity was attempted.

The non-symbiotic N_2 fixing microorganisms existing in the pasturelands under investigation were isolated and identified to gain more knowledge of their occurrence in the environment.

REVIEW OF LITERATURE

Early Investigations

The study of N_2-fixation had its beginning when Jodin (1862) reported evidence of organisms capable of metabolizing elemental nitrogen. However, it was years later that Winogradsky (1895) isolated and identified a N_2-fixing strain of anaerobic baccili (Clostridium butyricum). Following Winogradsky, Beijerinck (1901) isolated an aerobic bacteria which he gave the generic name Azotobacter. Diversed and intensive N_2-fixation studies ensued.

At the onset the mere ability of an organism to grow on "nitrogen free" media was considered proof of its N_2-fixing capacity. However, as refinement developed with more intensive research, this "proof" was nullified as evidence since small amounts of fixed nitrogen are always present in the media and ammonia could be absorbed from the air. Line and Loutit (1971) reported that Pseudomonas which survived ten serial transfers on nitrogen-deficient agar medium failed to demonstrate $N_2[C_2H_2]$ fixation. Similar results were found by Rice and Paul (1972).

Measurements of total nitrogen changes in the soil have been used to indicate non-symbiotic N_2-fixation (Parker, 1957; Moore, 1961). Problems associated with this method were the insensitivity of the conventional Kjeldahl analysis to detect net changes in nitrogen content which are usually small and obscurity of the quantity of nitrogen metabolized from N_2 from the quantity of nitrogen obtained from other sources.

Burris and Miller (1941) developed a sensitive tracer N^{15} technique

which provided a direct measurement of N_2 fixed. Studies have been performed using this method to evaluate non-symbiotic N_2-fixation in laboratory soil systems (Delwiche and Wijler, 1956; Brouzes, Mayfield and Knowles, 1971; Fehr, et. al., 1971). The cost of the isotope precludes its practicality for evaluation of in-situ fixation.

The acetylene-reduction test for the presence of nitrogenase, the enzyme complex responsible for N_2-fixation, was established by separate observations of Dilworth (1966) and Schollhorn and Burris (1967). Dilworth observed C_2H_2 to be reduced to C_2H_4 in an analogous fashion as N_2 is reduced to NH_3, and Schollhorn and Burris found that C_2H_2 inhibited N_2-fixation. The ease and sensitivity of this method has served as the second impetus to the voluminous N_2-fixation studies in the biochemical, microbiological, and agronomical realms. Of the latter, Stewart, et.al., (1967) were the first to develop a method of measuring in-situ fixation in soil which was later refined by Hardy (1968) to minimize soil sample alterations from field conditions. The acetylene-reduction test however remains as an indirect assessment of N_2 fixed, and calibration with N^{15} is necessary (Bergensen, 1970).

It is generally conceded that the contribution of non-symbiotic N_2-fixing microflora is small. The definition of significance is often dependent on the soil type, cultural practice of the soil, and the investigator's bias. Parker (1957) suggested that non-symbiotic fixation could be of considerable importance in the nitrogen economy of grassland soils, particularly those of fine texture. Similarly Jensen (1965) and Jurgensen (1973) contended that nitrogen gains may be significant in soils where organic matter is allowed to decompose. Steyn and Delwiche

(1970) concluded that N_2-fixation is not economically important in agronomic agriculture but when augmented with nitrogenous compounds in rainfall it may contribute significantly to the nitrogen balance in unfertilized range souls. Computation of a virgin Canadian grassland system showed more nitrogen would be added during the year through precipitation than biological fixation on a kg/ha basis (Paul, et al., 1971).

Non-symbiotic N_2-Fixation in Relation to Carbon

N_2-fixing microorganisms, except Cyanophyta and the anaerobic phototrophic bacteria, require exogenous carbohydrate as an energy source for metabolism. Although they are able to utilize an array of organic carbon compounds (Jensen, 1965; Kalininskaya, 1967), they are inefficient in terms of mg N fixed/g carbohydrate consumed and they must compete vigorously against other soil microflora for the available carbon compounds.

In numerous studies (Spiff and Odu, 1972; Delwiche and Wijler, 1956; Bjalfve, 1962; Change and Knowles, 1965; Rice, et al., 1967) supplementing the soil system with a carbon source demonstrated an increase in the N_2-fixing rate. The "availability" of the carbon source to the microorganisms has been shown to differ in its capacity to support growth and fixation. Delwiche and Wijler (1956) reported only a slight increase in fixation with the incorporation of grass cuttings, straw, or alfalfa, but a substantial increase with the addition of glucose or sucrose (Fehr, et al., 1972; Okafor and MacRae, 1973). Effects of kind and concentration of available carbon have also been investigated. A low concentration (1.5%) of glucose supported more rapid development of

nitrogenase activity than did a similar concentration of mannitol (O'Toole and Knowles, 1973); however, Okafor and MacRae (1973) observed a depression of C_2H_4 production at a glucose concentration of 2.5%.

It can be concluded from these laboratory studies that carbon is limited in natural environments and may be the rate determining factor of N_2-fixation. However, Brouzes, et al., (1969) found no consistent response in fixation with incorporation of glucose in acid forest soils.

Non-symbiotic N_2-Fixation in Relation to Nitrogen

High nitrogen contents of the soil diminishes the ecological advantage N_2-fixing microorganisms possess over the other soil microflora in their competition for carbon sources. The N_2-fixing process reduces N_2 to NH_3 and the presence of free NH_3 represses nitrogenase activity. Nitrate studies in the field have shown that when the soil was devoid of NO_3-N a 10% increase in soil nitrogen was recorded while 200 ppm NO_3-N in the soil was sufficient to prevent any gains (Greenland, 1959). Delwiche and Wijler (1956) found that nitrate concentration greater than 0.10-0.15 meq/ 100 g soil suppressed N_2-fixation by Azotobacter. In most natural ecosystems the levels of combined nitrogen are insufficient to inhibit fixation (Stewart, 1969).

Non-symbiotic N_2-Fixation in Relation to C:N Ratio

Most N_2-fixing microorganisms require carbon sources, and high nitrogen content removes their ecological advantage over the other soil microflora; therefore, the carbon:nitrogen ratio of the soil becomes an important factor in the soil ecology of heterotrophic N_2-fixing bacteria.

Advantageous conditions for fixation would then be a high carbon concentration and a low nitrogen concentration, or a high C:N ratio (Alexander, 1967). This was shown by Bremmer and Shaw (1958) who found maximum losses of nitrogen (denitrification) when the C:N ratio was low (2 to 3) while at higher ratios N_2-fixation occurred. Measuring the organic matter and total N content, Moore (1963) recorded fixation at C:N ratios of 11 and 12 but no fixation at a ratio of 10.

Non-symbiotic N_2-Fixation in Relation to Soil Moisture

Microorganisms like all other living things require water for metabolism. It has been found that increasing the soil moisture content increased C_2H_2 reduction (Okafor and MacRae, 1973). Similar results were obtained in other laboratory studies. Stefanson (1972) found no acetylene reduction at an average soil water content below 16% in an Urrbrae soil; however, about 16% reduction increased exponentially in a pasture soil and linearly in a crop soil. In a field study Steyn and Delwiche (1970) observed little or no fixation when the moisture content of an unirrigated site was below the permanent wilting point of the soil. Koch and Oya (1974) found good correlation between C_2H_2 reduction rate and soil moisture content. Reasons for increased C_2H_2 reduction with increasing soil moisture content could be due to a more favorable moisture level for microbial metabolism or the effect of higher moisture content upon soil aeration.

Non-symbiotic N_2-Fixation in Relation to Temperature

Free-living N_2-fixing microorganisms have been isolated from Antarctic lakes and Arizona deserts. These diverse temperature niches

indicate fixation is not governed by a rigid temperature regime. In his discussion of ecological factors, Jensen (1965) set the minimum temperature at 6 C with an optimum at 25-30 C. Since less N was fixed at 35 C. Fehr, et al., (1972) felt the optimum temperature range should be 15-25 C.

Non-symbiotic N_2-Fixation in Relation to Oxygen

N_2-fixing microorganisms that are strict anaerobes or facultative anaerobes fix nitrogen only under anaerobic conditions while aerobic microorganisms require oxygen for metabolism.

The efficiency of aerobic organisms to perform fixation is highly dependent on the oxygen level present. It is well documented that Azotobacter increases in its efficiency with decreases in pO_2; similar results have been obtained for blue-green algae (Stewart and Pearson, 1970). High pO_2 levels also inhibit N_2-fixation by its competition as terminal electron acceptors.

Brouzes, Mayfield and Knowles (1971) found that an anaerobic soil assay following anaerobic preincubation had a greater N_2-fixing activity than did an aerobic soil assay following aerobic preincubation. However, O'Toole and Knowles (1973) observed greater C_2H_2 reducing activity when their sandy loam soil was assayed under ambient conditions than when assayed under aerobic or anaerobic conditions. Whether the gas phase in the head-space of the soil container was aerobic or anaerobic, Okafor and MacRae (1973) found no effect upon C_2H_2 reduction.

Non-symbiotic N_2-Fixation in Relation to N_2-Fixing Microorganisms and Their Population

In the past most investigations on non-symbiotic N_2-fixing

microorganisms have been concentrated on Azotobacter and to a lesser extent, on Clostridium, which were thought to be the only N_2-fixing bacteria. Today many strains belonging to genera other than these two have demonstrated N_2-fixation and this property has been considered quite universal in the procaryotic world. (See Stewart (1969) or Nutman (1971) for a list of non-symbiotic N_2-fixing microorganisms.)

Azotobacter have been isolated from different locations and their sporadic occurrence appears to be governed by soil acidity and nutrient status. On the other hand, Clostridium have been found to be ubiquitous in the soil. Becking (1961) performed a global investigation and found Beijerinckia to be geographically restricted to the tropics due to laterization of the soil. Strydom (1965) found that soil acidity (pH less than 6.2) rather than lateritic soil type to be the major factor determining the presence of this organism.

Less investigations have been performed on the occurrence of the other non-symbiotic N_2-fixing bacteria. In acid forest soils Jurgensen and Davey (1971) found a predominance of Bacillus polymyxa and no aerobic N_2-fixing bacteria. From some New Zealand tussock-grassland soils Line and Loutit (1971) isolated Bacillus circulans, B. polymyxa, Enterobacter aerogenes, Klebsiella pneumoniae, and Escherichia intermedia. From some Hawaiian pastureland Koch and Oya (1974) isolated Enterobacter aerogenes, E. cloacae, Klebsiella pneumoniae, and an Achromobacter spp. Their individual populations in the soils were not determined.

N_2-fixing blue-green algae occur in moist slightly alkaline soil habitats where there is a shortage of combined nitrogen. They are found to be more abundant in tropical areas than temperate regions, however they have been isolated from frigid Arctic regions to desert

crusts in the United States. A review of algal fixation has been presented by Stewart (1970). Kobayashi and Haque (1971) have found photosynthetic bacteria contributed significantly to soil fertility. Studies on the ecology of this group of organisms are scarce and more investigations are necessary to assess their significance to soil nitrogen.

Nitrogenase activity was related to the number of N_2-fixing Clostridium in Rice and Paul's (1972) waterlogged soil amended with straw. Chang and Knowles (1965), Koch and Oya (1974), and Hanson (1974) also found a good relationship between nitrogenase activity and N_2-fixing population.

MATERIALS AND METHODS

Description of the Soils

Five different soil types, all extensively devoted to pasture-land on Maui, Hawaii, were sampled bi-weekly from January to June, 1974 (Table 1).

The soil series of Makena, Kaimu, and Kaipoioi (Figure 1) transect the leeward slope of Haleakala while the Haiku series represents a sample of the windward slope. The Haliimaile series were analyzed to discern the effects of a fertilized versus an unfertilized plot in their respective capacities to fix nitrogen. A description of the soils is presented in Table 2.

Six random core samples (15 cm deep) from a 100 square feet area of each site were composited and stored in doubled polyethylene bags. Two days were allowed for shipment to Oahu.

In-Vitro N_2-Fixation Capacity

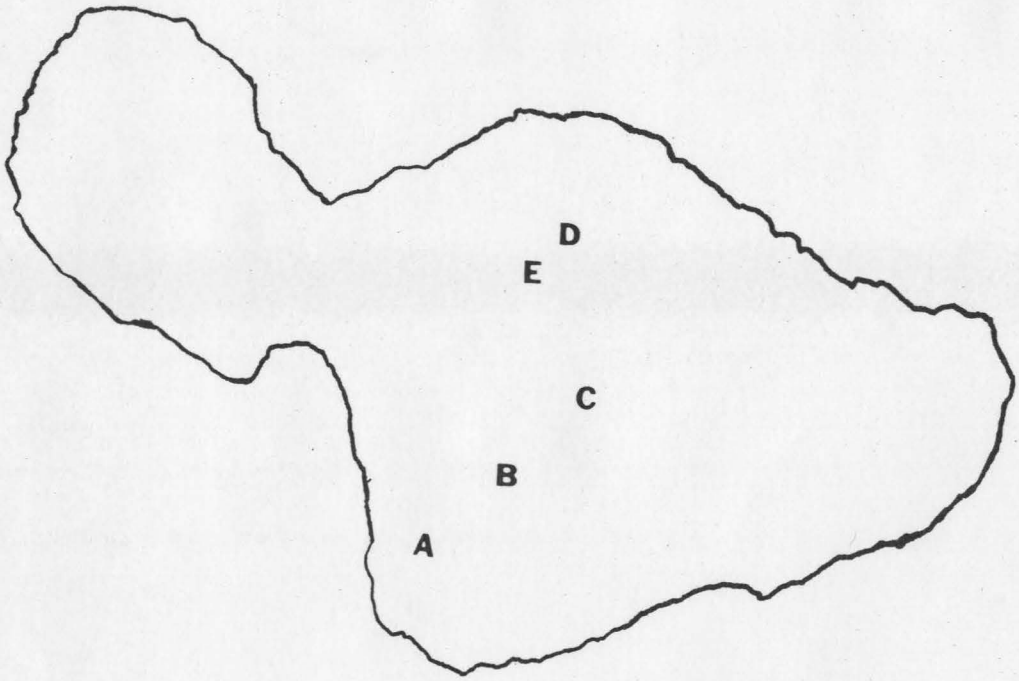
In-vitro N_2-fixation studies were conducted in duplicate with controls to detect any ethylene-producing bacteria. Five grams of soil were placed in 38 cc serum bottles in the laboratory. For the analysis of acetylene reduction under anaerobic conditions, the bottles were immediately serum-capped, evacuated and flushed with nitrogen gas three times. For the aerobic reduction of acetylene, the bottles were serum-capped without flushings with nitrogen gas. All bottles, with the exceptions of the controls, were injected with 2.5 cc acetylene.

Samples of the gas phase (0.5 cc) were injected into a Varian Aerograph Model 600D single column gas chromatograph incorporating a

TABLE 1. BI-WEEKLY SOIL SAMPLING

Sample Number	Date
1	January 7, 1974
2	January 21, 1974
3	February 4, 1974
4	February 19, 1974
5	March 4, 1974
6	March 18, 1974
7	April 1, 1974
8	April 15, 1974
9	April 29, 1974
10	May 13, 1974
11	May 27, 1974
12	June 10, 1974

FIGURE 1. MAUI, HAWAII



A Makena
B Kaimu
C Kaipoioi
D Haiku
E Haliimaile

TABLE 2. SOIL DESCRIPTION

	A	B	C	D	F	U
Subgroup & Great Group	Aridic Haplustols	Typic Tropofolists	Typic Dystrandepts	Orthoxic Tropohumults	Ustoxic Humitropepts	
Family	coarse-loamy, ashy, isohyperthermic	euic, isohyperthermic	medial isomesic	clayey, ferritic, isothermic	fine, kaolinitic, isothermic	
Series	Makena	Kaimu	Kaipoioi	Haiku	Haliimaile	
Elevation (km)	0.15	0.60	1.29	0.30	0.52	
Annual Rainfall (cm)	25-50	76-127	76-114	178-305	152-229	
MAST (C)	24	20	13	21	21	
pH	6.84	6.93	6.43	5.44	6.27	6.39
Vegetation	Stenotaphrum cenchras (Buffel)	Pennisetum clandestinum (Kikuyu)	Pennisetum clandestinum (Kikuyu)	Digitaria decumbens (Pangola)	Pennisetum clandestinum (Kikuyu)	Sporobolus capensis (Rattail)

hydrogen flame ionization detector and a recorder for the detection of acetylene and ethylene. Operating conditions were as follows: a stainless steel column 5 feet in length and 1/8 inch in diameter packed with Pora-Pak "R" of 80-100 mesh; pure nitrogen (carrier gas) passed through the column at a flow rate of 25 ml per minute; column temperature was 45 C. The ethylene and acetylene peaks from the column were identified by comparison of retention times with those of known standard samples of gas.

The amount of ethylene produced was recorded from a standard curve and its conversion to the quantity of nitrogen fixed was computed by incorporating a theoretical 3:1 ratio of acetylene to nitrogen.

Analysis of Environmental Factors

Each soil sample was analyzed as to the following: (A) pH: a 1:2 soil-water ratio was employed and the acidity determined on a Corning pH meter; (B) soil moisture content was determined gravimetrically; (C) carbon concentration was determined by the Walkey-Black method; (D) nitrogen concentration was determined by the micro-Kjeldahl method; (E) nitrate concentration was determined with the Orion Nitrate Specific Ion Electrode (Model 92-07); (F) soluble carbon concentration was determined by a modified method (Balasubramanian, 1974) of Perrier and Kellogg, (1960).

Rainfall and air temperature measurements were obtained through the Climatological Data published by the U.S. Department of Commerce.

Microbial Population Determination and Identification

The N_2-fixing bacterial population was determined by the most probable number (MPN) method (American Public Health Association, 1955)

under both aerobic and anaerobic conditions in 38 cc serum bottles. Three 10-fold dilutions of the soil sample were examined with 5 bottles representing each dilution. Pre-incubation was done at 30 C for 36 hours. Bottles were then incubated for an additional 24 hours in the presence of acetylene, followed by analysis for ethylene. If ethylene production was demonstrated, the bottle was scored as positive. The medium employed was: glucose 10 g; $MgSO_5.7H_2O$ 0.5 g; $CaCO_3$ 1.0 g; NaCl 0.01 g; $FeSO_4.7H_2O$ 0.004 g; $Na_2MoO_4.2H_2O$ 0.005 g; $ZnSO_4.7H_2O$ 0.005 g; $CuSO_4.5H_2O$ 0.004 g; biotin 5 ug; p-aminobenzoic acid 10 ug; yeast extract 0.1 g; K_2HPO_4 0.68g; distilled water to 1 liter (pH adjusted to 7.2).

Isolation of N_2-fixing bacteria was conducted, aerobically and anaerobically, through direct plating of soil particles on the previously described media and enrichment broths with 1% pyruvate or 1% mannitol as carbon sources in standard mineral media: Na_2HPO_4-KH_2PO_4 0.02M; $MgSO_4.7H_2O$ 0.2 g; $CaCl_2$ 0.01 g; Ferric EDTA 0.1 ml; $ZnSO_4.7H_2O$ 0.5 mg; $MnSO_4.H_2O$ 0.5 mg; $CuSO_4$ 0.1 mg; cobalt nitrate 0.1 mg; sodium borate 0.1 mg; sodium molybdate 2.0 mg; distilled water to 1 liter. After 5 days colonies were picked off the direct soil plates and streaked for isolation while 1 ml of the enrichment was streaked followed by re-streaking for pure colonies. The isolation of Beijerinckia was conducted according to Becking (1961). Every isolate was able to grow on the glucose with yeast extract agar.

Detection of blue-green algae in the soil samples was performed using the media of Allen and Stanier (1968) with the omission of sodium nitrate. Analysis for the presence of photosynthetic and sulfate-reducing bacteria shown to be capable of N_2-fixation was also performed on media utilized by Kobayashi and Haque (1971), and Reiderer-Henderson and Wilson (1970), respectively.

All bacteria isolated were grown on glucose with yeast-extract slants in 38 cc serum bottles. The bottles containing facultative anaerobic bacteria were serum-capped and evacuated and flushed with nitrogen gas while the aerobic bacteria had their serum bottles fitted with sponge plugs. It has been found that flaming flasks (bottles) for aseptic transfer introduces sufficient ethylene to give misleading results (Freebairn and Buddenhagen, 1964). The controls indicated that the evacuation and flushing of nitrogen gas removed all traces of ethylene for the anaerobic procedure followed and that after 1 hour of aerobic incubation (with the sponge plugs) ethylene had diffused out of the bottle.

Following a 24-hour growth period the isolates were tested for their ability to reduce acetylene to ethylene. The N_2-fixing isolates were then identified through a series of biochemical tests in conjunction with Skerman's Identification of Bacteria to their respective genera. Bergey's manual aided in the identification of the Escherichia, Enterobacter, and Achromobactor, ssp. while the Klebsiella, Azotobactor, and Beijerinckia ssp. were identified by consulting Bascombe, et al., (1971) Stanier et al., (p. 612, 1970) and Becking (1974), respectively.

Multiple Regression Analysis

A step-wise multiple regression analysis (BMD 02R) was conducted using the computer to determine the environmental factor(s) governing in-vitro N_2-fixation rates. The factors analyzed were total soil nitrogen concentration, total carbon concentration, C:N ratio, water-soluble carbon concentration, moisture content, nitrate concentration, temperature, and N_2-fixing population. The squares and square roots of soluble carbon concentration and moisture content were included to detect any curvilinear

relationships which may exist between in-vitro fixation and soil carbon concentration and moisture. The F level for inclusion and deletion were 1.000 and 0.500, respectively. Variables that contributed less than 10% to the coefficient of determination were omitted in the final regression equations.

RESULTS

N_2-Fixing Rates

Fixation rates are reported in Tables 3 (aerobic) and 4 (anaerobic). No ethylene production were detected in the controls indicating the absence of ethylene-producing bacteria. For all soils except the Makena series nitrogenase activity was higher under an anaerobic condition than aerobic. The Haiku soil displayed the highest rate of C_2H_2-reduction while the Makena soil had the lowest rate.

Calculated annual rates (Table 5) under aerobic conditions show a maximum amount of 2.0 Kg N/ha in the Haiku soil. The Haliimaile and Kaipoioi soils had comparable rates of 1.4, 1.5, and 1.6, respectively. Lowest annual rates of 0.9 and 1.0 Kg N/ha were computed for the Makena and Kaimu soils. All fixation rates are presented on a dry soil weight basis.

Environmental Factors

In every soil large fluctuations in the N_2-fixing populations (Table 6 and 7) can be observed. The Makena soil generally had the lowest population and contrary to the other samples its aerobic population predominated. The highest numbers of N_2-fixers were recorded in the Haliimaile fertilized plot. Total bacterial counts (Appendix Table 20) were determined on soil extract agar to observe the total microflora activity during each sample.

Soil moisture content (Table 8) tended to increase with rainfall (Appendix Table 18). Although the Kaipoioi soil had the highest moisture percentages, its consistency is very friable and the actual amount of available water to the microorganisms may be masked. The permanent

TABLE 3. AEROBIC IN-VITRO N_2-FIXATION (nmoles N_2/g day-1)

Sample No.	Makena	Kaimu	Kaipoioi	Haiku	Haliimaile Fertilized	Haliimaile Unfertilized
1	----	----	----	.141	----	----
2	.212	.068	.103	.089	.066	----
3	----	----	----	.106	----	----
4	----	----	.105	.102	.042	.033
5	.030	.058	.042	.067	.081	.064
6	.067	.157	.174	.496	.224	.267
7	.144	.178	.184	.168	.357	.305
8	.169	.143	.146	.194	.209	.177
9	.082	.212	.185	.225	.170	.474
10	.069	.125	.136	.094	.177	.059
11	----	----	.081	.087	.114	.122
12	.130	.153	.317	.368	.205	.104
MEAN	.075	.091	.123	.178	.137	.134

TABLE 4. ANAEROBIC IN-VITRO N_2-FIXATION (nmoles N_2/g day-1)

Sample No.	Makena	Kaimu	Kaipoioi	Haiku	Haliimaile Fertilized	Haliimaile Unfertilized
1	----	----	.160	.594	.153	.168
2	.082	----	.017	.228	.020	----
3	.012	.128	.057	.714	.576	.409
4	----	.096	.048	.550	.065	.136
5	.038	.116	.094	.029	.247	.114
6	.081	.181	.203	.288	.221	.335
7	.171	.154	.198	.242	.506	.270
8	.106	.187	.196	.484	.217	.183
9	.092	.337	.206	1.255	.332	.576
10	.046	.104	.190	.553	.478	.299
11	----	----	.112	.057	.222	.339
12	.103	.168	.327	.638	.212	.144
MEAN	.058	.123	.151	.469	.270	.248

TABLE 5. CALCULATED ANNUAL N_2-FIXATION IN SOIL

Soil Sample	Aerobic (Kg N/ha Year-1)	Anaerobic (Kg N/ha Year-1)
Makena	0.9	0.7
Kaimu	1.0	1.4
Kaipoioi	1.6	3.1
Haiku	2.0	5.4
Haliimaile: Fertilized	1.4	1.7
Haliimaile: Unfertilized	1.5	2.8

TABLE 6. AEROBIC N_2-FIXING POPULATION (cells/g soil)

Sample No.	Makena	Kaimu	Kaipoioi	Haiku	Haliimaile Fertilized	Haliimaile Unfertilized
1	110	130	680	1,700	170,000	330
2	220	1,700	230	2,400	160,000	790
3	130	1,700	9,200	2,200	240,000	16,000
4	20	790	16,000	1,100	130,000	24,000
5	33	2,400	1,700	23	7,800	1,700
6	350	460	4,700	2,400	2,100	200
7	350	1,700	1,400	920	24,000	14,000
8	350	250	4,900	540	2,200	200
9	920	790	2,700	110	35,000	130
10	48	790	780	2,400	17,000	9,200
11	350	1,300	54,000	350	92,000	330
12	540	9,200	4,900	350	2,300	490
MEAN	285	1,768	8,432	2,858	73,533	5,614

TABLE 7. ANAEROBIC N_2-FIXING POPULATION (cells/g soil)

Sample No.	Makena	Kaimu	Kaipoioi	Haiku	Haliimaile Fertilized	Haliimaile Unfertilized
1	45	78	28,000	5,400	3,200	2,200
2	490	1,100	200	2,300	240,000	1,100
3	170	790	1,700	2,200	24,000	2,200
4	20	230	9,200	180	220,000	7,900
5	170	490	400	33	4,500	17,000
6	540	78	1,700	1,600	1,400	450
7	170	490	1,300	49	11,000	610
8	920	4,300	5,400	350	1,100	780
9	170	310	790	79	780	68
10	33	640	9,200	5,400	14,000	2,400
11	110	5,400	2,800	180	160,000	1,100
12	130	1,100	790	280	2,600	40
MEAN	247	1,250	5,123	1,504	56,882	2,987

wilting point of each soil was not determined.

Table 9 shows the total nitrogen content of the soil as determined by the micro-Kjeldahl method and Table 10 illustrates the NO_3-N concentrations. The Kaipoioi soil had the highest nitrogen content during the sampling period while the Haiku soil displayed the lowest amount. The nitrate concentration was highest in the Makena soil sample and on May 13, 1974, had as much as 3.53 meq/100 gram dry soil.

Total organic carbon (Table 11) was highest in the Kaipoioi soil (av 15.81%) and the lowest in the Haiku series (av 2.95%). Interestingly both Kaimu and Haliimaile (unfertilized) plots increased significantly in organic carbon content on March 4, 1974, and remained high till the conclusion of sampling. The C:N ratios (Table 12) varied since fluctuations in total carbon had occurred. On the average 15.15 for the Haliimaile fertilized plot was the highest and 10.68 for the Makena soil was the lowest. Soluble carbon content (Table 13) in the soils varied with sampling. Based on their averages during the period of sampling the order of descending soluble carbon content (mg/100 g soil) was Kaipoioi (61.7), Haliimaile fertilized plot (59.3), Kaimu (59.1), Makena (54.4), Haiku (43.3), and the Haliimaile unfertilized plot (41.3).

Temperature and rainfall data were recorded from the stations closest to each site and are tabulated in Appendix Tables 18 and 19.

TABLE 8. SOIL MOISTURE CONTENT (%)

Sample No.	Makena	Kaimu	Kaipoioi	Haiku	Haliimaile Fertilized	Haliimaile Unfertilized
1	40.06	84.66	113.13	33.36	82.90	80.22
2	31.96	67.91	95.29	34.16	79.02	72.04
3	32.52	74.93	99.67	34.00	81.02	74.60
4	31.83	54.67	117.54	42.48	104.06	89.88
5	26.40	63.25	78.87	34.47	85.96	73.17
6	32.20	58.20	92.40	35.07	79.00	73.26
7	36.46	65.37	90.46	30.80	81.22	69.68
8	30.55	53.64	77.40	28.18	66.44	51.58
9	38.77	90.56	101.79	33.90	93.56	77.06
10	20.53	45.42	65.32	29.48	77.58	64.72
11	14.32	43.50	73.85	30.46	66.96	54.95
12	19.94	42.07	81.39	30.63	50.62	35.78
MEAN	29.63	62.02	90.59	33.08	79.03	68.12

TABLE 9. TOTAL NITROGEN CONTENT (%)

Sample No.	Makena	Kaimu	Kaipoioi	Haiku	Haliimaile Fertilized	Haliimaile Unfertilized
1	.50	.75	1.14	.26	.95	.73
2	.43	.70	1.25	.17	.88	.72
3	.40	.74	1.25	.26	.92	.73
4	.43	.80	1.23	.26	.92	.72
5	.42	.73	1.19	.26	.96	.77
6	.43	.63	1.32	.24	.89	.75
7	.47	.75	1.25	.26	.92	.75
8	.42	.75	1.18	.26	.91	.77
9	.37	.77	1.09	.25	.90	.68
10	.40	.58	1.18	.25	.92	.74
11	.49	.88	1.15	.29	.82	.83
12	.52	.83	1.17	.27	.95	.73
MEAN	.44	.74	1.20	.25	.91	.74

TABLE 10. NITRATE-NITROGEN (meq/100 grams dry soil)

Sample No.	Makena	Kaimu	Kaipoioi	Haiku	Haliimaile Fertilized	Haliimaile Unfertilized
1	.06	.07	.03	.02	.03	.02
2	1.53	.07	.02	.02	.01	.02
3	.72	.06	.04	.01	.07	.02
4	2.89	.05	.02	.01	.02	.01
5	2.74	.16	.32	.01	.35	.02
6	1.04	.05	.03	.02	.02	.02
7	1.80	.38	.02	.02	.05	.02
8	1.80	.20	.07	.02	.04	.03
9	.89	.76	.05	.03	.05	.03
10	3.53	.13	.04	.02	.03	.01
11	2.41	.44	.05	.03	.05	.04
12	1.37	.26	.04	.03	.04	.02
MEAN	1.73	.18	.06	.02	.06	.02

TABLE 11. TOTAL ORGANIC CARBON (%)

Sample No.	Makena	Kaimu	Kaipoioi	Haiku	Haliimaile Fertilized	Haliimaile Unfertilized
1	5.52	6.72	15.38	3.02	6.31	5.82
2	3.71	4.71	13.86	2.76	10.10	7.41
3	4.16	6.12	12.57	1.93	6.70	7.52
4	4.46	5.72	14.85	2.10	10.34	8.34
5	4.52	10.99	16.00	3.61	12.50	13.04
6	4.94	10.22	18.61	3.68	17.42	17.90
7	4.78	11.24	17.14	3.91	17.84	15.22
8	4.74	12.06	17.07	3.74	15.83	17.44
9	4.18	11.13	15.76	3.47	16.66	12.75
10	4.44	9.50	15.16	3.71	14.54	13.60
11	5.70	12.63	16.62	3.97	18.20	15.08
12	5.22	11.05	16.68	3.06	18.33	12.56
MEAN	4.70	9.34	15.81	2.95	13.73	12.22

TABLE 12. CARBON:NITROGEN RATIOS

Sample No.	Makena	Kaimu	Kaipoioi	Haiku	Haliimaile Fertilized	Haliimaile Unfertilized
1	11.04	8.96	13.49	11.62	6.64	7.97
2	8.63	6.73	11.09	16.24	11.48	10.29
3	10.40	8.27	10.06	7.42	7.28	10.30
4	10.37	7.15	12.07	8.08	11.24	11.58
5	10.76	15.05	13.45	13.88	13.02	16.94
6	11.49	16.22	14.10	15.33	19.57	23.87
7	10.17	14.99	13.71	15.04	19.39	20.29
8	11.29	16.08	14.47	14.38	17.40	22.65
9	11.30	14.45	14.46	13.88	18.51	18.75
10	11.10	16.38	12.85	14.84	15.80	18.38
11	11.63	14.35	14.45	13.69	22.20	18.17
12	10.04	13.31	14.26	11.33	19.29	17.21
MEAN	10.68	12.67	13.21	12.97	15.15	16.37

TABLE 13. SOLUBLE CARBON (mg/100 grams dry soil)

Sample No.	Makena	Kaimu	Kaipoioi	Haiku	Haliimaile Fertilized	Haliimaile Unfertilized
1	38.25	38.50	36.95	38.35	32.10	39.75
2	58.10	61.05	55.85	65.38	37.82	60.12
3	52.50	63.00	57.00	49.08	81.40	45.55
4	45.82	63.05	59.10	52.42	53.75	44.12
5	52.35	60.42	55.25	47.75	45.75	40.32
6	48.12	41.88	68.90	40.32	58.95	35.85
7	51.78	49.65	53.00	52.65	80.65	34.75
8	59.00	47.75	64.38	36.35	58.95	44.90
9	50.75	43.00	60.85	28.40	49.68	30.42
10	51.10	83.00	80.30	36.50	39.65	72.00
11	73.72	77.88	66.45	35.02	74.50	18.52
12	72.10	79.55	82.50	37.50	98.90	29.10
MEAN	54.44	59.06	61.71	43.31	59.34	41.28

Multiple Regression Analysis

The step-wise multiple regression study (Table 14) revealed that different environmental factors controlled fixation rates under aerobic and anaerobic conditions in the same soil and that the determining factors varied amongst the different soil types.

Major factors affecting aerobic nitrogen fixation rates in the Makena soil were its C:N ratio and nitrogen-fixing population. The C:N ratio explained 28% of the variation in fixation while the population accounted for an additional 19%. Under anaerobic conditions, the nitrogen-fixing population was the only factor entering the regression equation and explained 26% of the total variation. Variance analysis showed that the aerobic and anaerobic fixation rates were not significantly different at the 90% confidence level. Therefore a combined regression analysis was performed to construct a regression equation and the coefficient of determination for this equation was found to be 35%. Population and C:N ratio were the contributing factors.

The Kaimu soil showed its aerobic fixation rate controlled primarily by its C:N ratio and the coefficient of determination for its equation was 42%. Under anaerobic conditions nitrate concentration and temperature were the major factors and explained 5% of the variation. Like the Makena soil, the aerobic and anaerobic fixation rates were not significantly different. Factors explaining the variation for aerobic and anaerobic N_2-fixation were the C:N ratio and the moisture content and the coefficient of determination was 42%.

TABLE 14. MULTIPLE REGRESSION EQUATIONS

			R^2
Makena	aerobic:	$\hat{y}$ = 0.60447 - 0.05295 C:N + 0.00013 population	.47
	anaerobic:	$\hat{y}$ = 0.03515 + 0.00009 population	.26
	combined:	$\hat{y}$ = 0.3822 + 0.00011 population - 0.3218 C:N	.35
Kaimu	aerobic:	$\hat{y}$ = 0.00359 + 0.01519 C:N + 0.00002 moist^2	.42
	anaerobic:	$\hat{y}$ = 0.96606 + 0.30490 NO_3 - 0.05121 temperature	.54
	combined:	$\hat{y}$ = - 0.1611 + 0.01598 C:N + 0.00002 moisture^2	.42
Kaipoioi	aerobic:	$\hat{y}$ = - 0.35228 + 0.00003 soluble carbon^2 + 0.02157 carbon	.61
	anaerobic:	$\hat{y}$ = 0.11161 + 0.03577 C:N + 0.00016 soluble carbon^2 - 0.01737 soluble carbon	.78
	combined:	$\hat{y}$ = - 0.38113 + 0.03064 C:N + 0.00003 soluble carbon^2	.60
Haiku	aerobic:	$\hat{y}$ = - 1.39929 - 0.06896 temperature + 12.4966 NO_3	.56
	anaerobic:	$\hat{y}$ = 9.95918 - 0.68554 soluble carbon$^{1/2}$ - 0.08590 C:N - 12.97688 nitrogen - 0.20851 carbon	.94
Haliimaile Fertilized	aerobic:	$\hat{y}$ = - 0.13910 + 0.02011 carbon	.70
	anaerobic:	$\hat{y}$ = 0.01632 - 0.00003 population + 0.277 temperature	.56
Unfertilized	aerobic:	$\hat{y}$ = - 0.38024 + 0.13472 C:N - 0.15348 carbon + 8.49211 NO_3	.13
	anaerobic:	$\hat{y}$ = 0.28185 - 0.00001 population	.52

The carbon content of the Kaipoioi soil expressed the strongest influence on its rates. Under aerobic conditions soluble carbon concentration squared explained 47% of the variation while the total carbon concentration explained an additional 13%. The major factors under anaerobic conditions were soluble carbon concentration, soluble carbon concentration squared and C:N ratio (coefficient of determination = 78%). A combined analysis of both aerobic and anaerobic rates with their corresponding factirs showed the C:N ratio and the soluble carbon concentration squared as key factors in fixation. The coefficient of determination of this combined equation was 60%.

Aerobic and anaerobic fixation rates in the Haiku soul were significantly different and thus no combined equation was computed. While temperature and nitrate concentration had the greatest effect on the aerobic rates (coefficient of determination = 56%), nitrogen concentration, carbon concentration, C:N ratio, and the square root of the soluble carbon concentration influenced anaerobic fixation (coefficient of determination = 94%).

The Haliimaile plost were analyzed to discern the effects of a fertilized versus an unfertilized plot. The results showed different environmental factors controlling their fixation capacity. The fertilized plot had its aerobic fixation rate controlled (coefficient of determination = 70%) by carbon concentration and its anaerobic fixation rate (coefficient of determination = 56%) by population and temperature. The C:N ratio, carbon concentration and nitrate concentration effected the unfertilized plot's rate under aerobiosis with a coefficient of determination of 13% for its equation while under anaerobiosis population explained 52% of the variation in fixation. The aerobic and anaerobic

rates of both plots were found to be significantly different.

General regression equations for in-vitro nitrogen fixation rates under aerobiosis and anaerobiosis were computed, incorporating data from all six soil samples. The aerobic and anaerobic assays were computed separately since the fixation rates were found to be significantly different in the Haiku and Haliimaile (both fertilized and unfertilized plots) soils. The regression equations obtained were:

Under Aerobic Condition:

$y = - 0.07378 + 0.01457$ C:N (coefficient of determination = 27%)

Under Anaerobic Conditions:

$y = 0.44990 - 0.12294\ NO_3 - 0.26131$ Nitrogen (coefficient of determination = 22%).

Isolation and Identification of N_2-Fixing Bacteria

Approximately 200 strains of bacteria (aerobic and anaerobic) were capable of growth after five serial transfers on nitrogen deficient agar. When these isolates were examined for C_2H_2 reducing ability, it was found that only 75 had this ability. The 75 isolates were carefully examined for their gram stain, motility, cell morphology, fermentation reaction if possible, and colony morphology on tetrazolium plates (Appendix Table 22) before they were ascribed into similar groupings. Resulting were five aerobic bacteria, three facultative anaerobic bacteria and one strictly anaerobic bacterium.

Of the five aerobic nitrogen-fixing bacteria, two were identified as belonging to the genus Azotobacter. Azotobacter choococcum and A. macrocytogenes were identified to their genus level with Skerman's Key to the Identification of Bacteria and to their species level consulting the table in Stanier, et al., (1970, p. 612).

Another two, Achromobacter xerosis and Beijerinckia fluminensis, were identified to their species level in conjunction with Bergey's Manual and Becking (1974), respectively. A. xerosis had the following characteristics: 0.5 X 1.0-1.5 um; oxidative metabolism; catalase +, gram -, motility +; urease +; NO_3-NO_2 +; capsules +; oxidase +; litmus milk alkaline; gelatin liquefication +; starch hydrolysis -; and casein hydrolysis -. B. fluminensis (0.75 X 1.5 um) had the characteristic highly refractile polar bodies, granular agar colony, and the formation of ascococci.

The fifth aerobic bacteria remained unidentified. It had the following characteristics: rod (1.5 X 2.5-5.0 um); oxidative metabolism; nonmotile; gram -; catalase +; urease +; spores -; capsules +; NO_3-NO_2 -;

litmus milk peptonization; oxidase -; gelatin hydrolysis +; starch hydrolysis +; and lipase -. It differs from Derxia gumnosa (Jensen, et al., 1960) in that it had a strictly oxidative metabolism, catalase +, and no red pigment formation.

The three facultative anaerobes were found to belong to the family Enterobacteriaceae. Their characteristics and biochemical reactions are recorded in Table 15. Klebsiella pneumoniae demonstrated acidic products of fermentation (methyl red +) and the absence of acetoin (Voges-Proskauer -). Although the results conflicted with Bergey's Manual, similar results have been found by Bascomb, et al., (1971) in their classification of Klebsielleae.

The strict anaerobe was observed to be a Clostridium spp. Although it was isolated in all six soils, no attempts were made to identify it to its species level. In Table 16 is illustrated the occurrence of N_2-fixing bacteria in the different soil systems.

Neither N_2-fixing blue-green algae nor photosynthetic bacteria was isolated from the six pasturelands. A plausible explanation by Stewart, et al., (1967) is that in well-established pasture insufficient light at the soil surface attributes to the lack of blue-green algae which would equally apply to the photosynthetic bacteria.

TABLE 15. CHARACTERISTICS OF FACULTATIVE N_2-FIXING BACTERIA

Test or Substrate	*Klebsiella pneumoniae*	*Enterobacter aerogenes*	*Escherichia intermedia*
Size (um)	0.5X.75-1.0	0.5-.75X1-2	0.25-0.50X1.0-1.5
Motility	-	+ (peritr.)	+ (peritr.)
Gram	-	-	-
Oxidase	-	-	-
Catalase	+	+	+
Methyl Red	+	+	+
Voges-Proskauer	-	+	+
Indole	-	+	-
Citrate	+	+	+
Fermentation: Glucose	AG	AG	AG
Cellobiose	AG	AG	AG
Sucrose	AG	AG	AG
Lactose	AG	AG	AG
Sorbitol	AG	A	AG
Urease	+	+	-
Gelatinase	-	-	-
Amylase	-	-	-
Lipase	-	+	-
Gelatin hydrolysis	+	-	-
Starch hydrolysis	+	-	-
H_2S production	-	-	-
Capsule	+	-	-
Litmus Milk	AC	AC	A
Lysine decarboxylase	+	+	-
Ornithine decarboxylase	-	-	-
Arginine decarboxylase	-	-	+
Phenylalanine deaminase	-	-	+
NO_3-NO_2	+	+	+
KCN	-	-	+

A = acid; G = gas; C = coagulation; peritr. = peritrichous

TABLE 16. OCCURRENCE OF NON-SYMBIOTIC N_2-FIXING BACTERIA IN PASTURELANDS

Makena:	Unidentified aerobe *Achromobacter xerosis* *Azotobacter chroococcum* *Clostridium* spp.
Kaimu:	Unidentified aerobe *Azotobacter macrocytogenes* *Klebsiella pneumoniae* *Enterobacter aerogenes* *Clostridium* spp.
Kaipoioi:	Unidentified aerobe *Clostridium* spp.
Haiku:	*Beijerinckia flumenensis* *Achromobactor xerosis* *Klebsiella pneumoniae* *Enterobactor aerogenes* *Clostridium* spp.
Haliimaile:	
Fertilized plot	Unidentified aerobe *Enterobactor aerogenes* *Escherichia intermedia* *Clostridium* spp.
Unfertilized plot	*Achromobactor xerosis* *Escherichia intermedia* *Clostridium* spp.

DISCUSSION

Non-symbiotic nitrogen fixation rates were generally greater when the soil was incubated under an anaerobic rather than aerobic headspace. Similar results have been reported by Brouzes, et al., (1971), Koch and Oya, (1974), and Chang and Knowles (1965). Anaerobiosis as typified in the method would occur in the fields if the atmosphere was completely devoid of oxygen or if the soil was saturated throughout the year. Since the soil samples are representatives of pastureland, where saturation seldom occurs for long periods of time, the aerobic rates of C_2H_2 reduction were more indicative of the actual field events.

For the Makena, Kaimu, Kaipoioi samples, the aerobic and anaerobic nitrogenase activities were not significantly different. Okafor and MacRae (1973) with similar results explained that under anaerobic conditions the absence of oxygen favored only the anaerobic and facultatively anaerobic N_2-fixers. However, in aerobically incubated soils, there exist microenvironments where anaerobiosis prevail. Soil texture and microbial respiration determine the degree of anaerobiosis persistence. Interestingly the Makena soil which is coarse-loamy in texture had a higher N_2-fixing population and C_2H_2 reducing activity under aerobic incubation in comparison to its population and activity under anaerobic conditions.

The annual fixation rates computed were highest for the wettest soil (Haiku 2.0 kg N/ha $year^{-1}$) and the lowest for the driest soil (Makena 0.9 kg N/ha $year^{-1}$). The extremely high NO_3-N concentration of the Makena soil appeared to be a limiting factor. Delwiche and Wijler (1956) found .10-.15 meq NO_3-N/100 g dry soil suppressed N_2-fixing acti-

vity. The Makena soil averaged 1.73 meq NO_3-N/100 g dry soil during the period of sampling. This may be the first report of a natural ecosystem to be sufficiently high in nitrate-nitrogen to suppress fixation.

The annual figures obtained are in reasonable agreement with estimates of Steyn and Delwiche (1970) of 2-5 kg N fixed/ha $year^{-1}$ in California soils and findings of Paul, Myers, and Rice (1971) of 1 kg N/ha $season^{-1}$ in Canadian grasslands. Spiff and Odu (1972) obtained slightly higher rates (11.4 kg N/ha $year^{-1}$) in unamended Nigerian soils (based on the ethylene production at 24 hours of incubation). In all soils, except the Haliimaile fertilized plot, no nitrogen fertilizer was added and they are usually low in nitrogen content. From personal communications with Dr. Paul Ekern (University of Hawaii) approximately 0.5 - 1.0 kg N/ha $year^{-1}$ are added to soils through precipitation in the Hawaiian Islands. Therefore, free-living N_2-fixing bacteria and precipitation contributed in approximately equal amounts to the total nitrogen input in unfertilized Hawaiian pasturelands.

The step-wise multiple regression analysis showed that different environmental factors controlled acetylene-reduction rates in the six pasturelands. In each soil the contributing factors also varied between aerobic and anaerobic conditions. This exemplified the complexity of the soil ecosystem. The individual multiple regression equations did not serve well as predictive models and demonstrated at most the uniqueness of each soil.

N_2-fixing population appeared in five regression equations and in one sample (Haliimaile fertilized plot under anaerobic conditions) a negative correlation was found. Although others (Hanson, 1974; Koch and

Oya, 1974; Jurgensen, 1971) have also recorded a good agreement between fixation rates and number of N_2-fixing bacteria, the method measured what must be considered the potential bacterial number. Five days in a rich carbohydrate broth with inorganic nutrients provided sufficient time for the germination of microcysts and spores.

The combined equations (6 soils) similarly revealed differences between aerobic and anaerobic limiting factors. While the C:N ratio (range 6.6-23.9) appeared to be governing the rate for aerobic fixation rate, the NO_3 and total N concentrations expressed a negative relationship with anaerobic C_2H_2 reduction rates. The increased fixation rate with increased C:N ratios have been postulated and/or proven by Alexander (1967), Bremmer and Shaw (1958), and Moore (1963). The negative effects of nitrate-nitrogen has been shown by Delwiche and Wijler (1956). Stewart (1964) has demonstrated stimulation of N_2-fixation by blue-green algae when the combined nitrogen was low.

The two Haliimaile soil samples were comparable in their N_2-fixing capacity. The fertilized plot was estimated to fix 1.4 kg N/ha $year^{-1}$ while the unfertilized plot was found to fix 1.5 kg N/ha $year^{-1}$. On March 21, 1974, 200 lbs urea/A was added which is equivalent to 104 kg N/ha, however no increase in total soil nitrogen content due to fertilization was possible to detect with the method utilized. The additional 1.4 kg N/ha $year^{-1}$ from biological fixation was not significant to the nitrogen balance of the fertilized soil. In most studies nitrogen was found to be inhibitory to N_2-fixation. However, Taha, et al., (1966, 1967) found combined nitrogen stimulated Azotobacter populations. During the 7th sampling (April 1, 1974) the aerobic N_2-fixing population increased to 24,000 cells/g dry soil and correspondingly the aerobic

fixation rate was amplified to 0.357 nmoles N_2/g day^{-1}. The fertilized plot had a higher microbial population and soluble carbon concentrations than the unfertilized plit but these two plots did not differ greatly in nitrate-nitrogen, total carbon, and C:N ratios. Although one incident indicated higher fixation rate with nitrogen fertilizer, no significant augmentations in nitrogen fixation with fertilization were observed when averaged over the 6-month sampling period. The benefit of nitrogen fertilization to free-living nitrogen-fixers may be the greater soluble carbon concentration resulting from increased plant growth (Jurgensen, 1973).

All of the identified isolates have been previously reported as free-living nitrogen-fixing bacteria. Azotobacter chroococcum, Azotobacter macrocytogenes, Klebsiella pneumoniae, Achromobacter zerosis, Enterobacter aerogenes, and Clostridium are well established as nitrogen-fixing bacteria while supporting evidences for some species of Escherichiae to fix nitrogen are being reported. Escherichia intermedia has been isolated and identified as a N_2-fixer in tussock-grassland (Line and Loutit, 1971). The unidentified aerobic nitrogen-fixer appeared similar in morphology to Derxia gumnosa (Jensen, et al., 1960); however, its mode of metabolism, presence of cytochrome c, and failure of red pigment formation suggested it was not the same organism.

A. macrocytogenes and A. chroococcum were isolated from the Kaimu and Makena samples, respectively. The pH of these soils are 6.8 for the Makena soil and 6.9 for the Kaimu soil. It has been shown that Azotobacter occur more frequently under alkaline conditions (Jensen, 1950 and Kaila, 1954). In contract the Beijerinckia are known to favor

an acidic condition (Stydom, 1965). The only Beijerinckia isolated was B. fluminensis in the Haiku soil with a pH of 5.4. The occurrence of Azotobacter and Beijerinckia in this study agreed with previous indications of the relationship between these bacteria and soil acidity. There were no indications of the facultative anaerobes being restricted by acidity. Clostridium was found to be ubiquitous in the soils.

SUMMARY AND CONCLUSIONS

The contributions of free-living N_2-fixing microorganisms to the nitrogen balance of six pasturelands on Maui, Hawaii, was studied using the acetylene-reduction technique. Highest aerobic fixation rate was computed in the Haiku soil at 2.0 kg N/ha $year^{-1}$. The lowest rate of 0.9 kg N/ha $year^{-1}$ observed in the Makena soil was believed to be a result of the high nitrate-nitrogen content in this soil. Fixation was 1.0 kg N/ha $year^{-1}$ in the Kaimu soil and 1.6 kg N in the Kaipoioi soil. Nitrogen fertilization showed no significant effects on N_2-fixation in spite of its large affect on the soluble carbon level. The fertilized plot was observed to fix 1.4 kg N/ha $year^{-1}$ while the unfertilized plot contributed 1.5 kg N/ha $year^{-1}$.

The step-wise multiple regression study revealed the complexity and variability of the soil ecosystem. Overall, the C:N ratio of the soils incubated under aerobic conditions appeared to be the most important factor governing fixation rate. The nitrate-nitrogen and total nitrogen expressed a negative relationship with the rate under anaerobic conditions.

Non-symbiotic N_2-fixing bacteria isolated and identified were Achromobacter xerosis, Azotobacter chroococcum, Azotobacter macrocytogenes, Klebsiella pneumoniae, Enterobacter aerogenes, Beijerinckia flumenensis, Eschrichia intermedia, and Clostridium. The occurrences of Azotobacter and Beijerinckia were observed to be governed by soil acidity.

In the Hawaiian pasturelands non-symbiotic N_2-fixation contributions are low. Nitrogen gains through precipitation are likely to be of the same order. Together, they may be significant to the nitrogen balance of the soil.

APPENDIX

APPENDIX TABLE 17. RAINFALL AND TEMPERATURE STATIONS

RAINFALL

Site	Station	Location Number*
Makena	Ulupalakua Ranch	250
Kaimu	Kula Sanatorium	267
Kaipoioi	Olinda	332
Haiku	Waikamoi Dam	336
Haliimaile	Haliimaile Dam	423

TEMPERATURE

Site	Station	Location Number*
Makena	Keawakapu Beach	260.2
Kaimu	Kula Sanatorium	267
Kaipoioi	Kula Research Center	-
Haiku	Kailua	446
Haliimaile	Haleakala R. S.	338

*Reference to climatological data published by U.S. Department of Commerce.

APPENDIX TABLE 18. RAINFALL (INCHES) BETWEEN SAMPLING PERIODS

Sample No.	Makena	Kaimu	Kaipoioi	Haiku	Haliimaile
1	5.58	5.99	2.28	2.63	1.65
2	2.58	5.43	5.12	4.98	2.58
3	4.66	9.21	6.52	0	7.03
4	2.27	1.19	0	0.21	1.30
5	0.44	1.14	0.22	1.87	0.11
6	1.85	1.43	2.91	6.42	0.91
7	0.75	1.87	0.76	3.52	2.11
8	1.87	4.76	1.94	1.44	3.22
9	2.30	2.43	3.84	21.89	5.64
10	1.74	0.25	0.78	6.67	0.52
11	0.54	0.60	0.58	4.54	0.66
12	1.84	2.40	0.79	3.28	0.02

APPENDIX TABLE 19. TEMPERATURE (C)

Sample No.	Makena	Kaimu	Kaipoioi	Haiku	Haliimaile
1	23	17	17	22	11
2	24	19	18	22	10
3	23	18	17	21	17
4	24	17	17	20	13
5	22	17	17	21	8
6	22	16	17	18	6
7	24	19	17	22	15
8	25	18	18	21	12
9	24	17	18	21	11
10	25	18	17	21	10
11	26	19	18	23	11
12	25	18	18	23	13

APPENDIX TABLE 20. TOTAL BACTERIA COUNT (X 10^6)

Sample No.	Makena	Kaimu	Kaipoioi	Haiku	Haliimaile Fertilized	Haliimaile Unfertilized
1	43	18	19	10	32	10
2	80	150	43	16	54	38
3	61	36	279	80	170	76
4	46	48	55	20	65	50
5	95	146	49	30	145	48
6	95	52	69	17	149	93
7	51	101	52	9	143	40
8	53	65	52	15	75	65
9	45	48	38	15	189	60
10	90	82	65	34	125	64
11	39	118	66	15	144	47
12	88	276	78	8	100	42

APPENDIX TABLE 21. pH

Sample No.	Makena	Kaimu	Kaipoioi	Haiku	Haliimaile Fertilized	Haliimaile Unfertilized
1	6.90	7.04	6.79	5.80	6.40	6.45
2	6.70	7.00	6.55	5.62	6.44	6.53
3	6.80	6.89	6.58	5.52	6.22	6.52
4	6.50	6.95	6.49	5.52	6.45	6.46
5	6.40	7.25	6.50	5.50	6.45	6.40
6	7.48	6.86	6.48	5.38	6.27	6.42
7	6.70	6.78	6.22	5.40	6.15	6.42
8	7.10	6.90	6.21	5.50	6.30	6.46
9	7.20	6.83	6.26	5.22	6.19	6.12
10	6.58	6.92	6.43	5.05	6.20	6.27
11	6.83	6.90	6.44	5.41	6.03	6.27
12	6.90	6.84	6.21	5.30	6.22	6.26

APPENDIX TABLE 22. TZC (TRIPHENYL TETRAZOLIUM CHOLORIDE) MEDIUM

Peptone	1.0%
Dextrose	0.5%
Agar	2.0%
TZC	.001%

TZC prepared separately as 1% solution and autoclave for 5 minutes.

APPENDIX TABLE 23. FERTILIZER APPLICATIONS (HALEAKALA DAIRY PADDOCK 19)

	lb/A of Urea
April 18, 1970	300
April 24, 1971	200
November 26, 1971	200
May 13, 1972	200
February 12, 1973	200
May 26, 1973	300
March 21, 1974	300

APPENDIX TABLE 24. FORMULA FOR CALCULATING ANNUAL RATES

$$\text{nmoles } N_2/g\ day^{-1} \times 14 = \text{nmoles } N_2/g\ (2\ weeks)^{-1}$$

$$\Sigma\ \text{nmoles } N_2/g\ (2\ weeks)^{-1} = \text{nmoles } N_2/g\ (24\ weeks)^{-1}$$

$$\text{nmoles } N_2/g\ (24\ weeks)^{-1} \times \frac{52}{24} \times (22.37 \times 10^8\ g/hec) = \text{nmoles } N_2/hec\ year^{-1}$$

$$\text{nmoles } N_2/hec\ year^{-1} \times \frac{28}{2} \times 10^{-6} = kg\ N/hec\ year^{-1}$$

APPENDIX TABLE 25. SOIL SAMPLE LOCATION

A. Makena: lat. 20° 39' 20" N. and long. 156° 25' 51" W.

B. Kaimu: lat. 20° 43' N. and long. 156° 21' 8" W.

C. Kaipoioi: lat. 20° 46' 5" N. and long. 156° 17' 41" W.

D. Haiku lat. 20° 54' 9" N. and long. 156° 18' 20" W.

E. Haliimaile: lat. 20° 50' 20" N. and long. 156° 18' 31" W.

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